REMARKS

By the foregoing amendment, claim 6 has been amended to render the claim language more definite. Applicants note that the amendment adds no new matter. Entry of the amendment is respectfully requested.

RESPONSE TO THE OFFICE ACTION

Formal Matters

Applicants note with appreciation that the Examiner has considered the documents submitted in the Information Disclosure Statement of February 22, 2007 by returning a signed and initialed Form PTO-1449.

Applicants also note that the Office Action indicates acknowledgment of the claim of foreign priority and receipt of all certified copies of priority documents.

Claim Rejections under 35 U.S.C. § 112

The Office Action rejects claim 6 under 35 U.S.C. § 112 as allegedly being indefinite for failing to comply with the definiteness requirement. In particular, the Action asserts that the phrase "a composition for a cosmetic, a medicament, a foodstuff, and/or a feed" is unclear. The Action raises two alternatives, i.e., whether said composition is to be used in a cosmetic, a medicament, a foodstuff, and/or a feed; or, whether the cosmetic, the medicament, the foodstuff, and/or the feed are the composition.

In response, Applicants submit the foregoing amendment to render claim 6 more definite. In view of the amendment, Applicants respectfully request withdrawal of the rejection.

Art-Based Claim Rejections (35 U.S.C. §§ 102(b) and 103(a))

The Action rejects claims 1-3, 5, and 8 under 35 U.S.C. § 102(b) as allegedly being anticipated by Trumbo et al. (Journal of Nutrition (1988), 118 (2), pp. 170-175). The Office Action asserts that Trumbo et al. anticipates claims 1-3 and 5 by allegedly disclosing a compound that falls within the scope of these claims. As for claim 8, the Office Action asserts that although Trumbo et al. does not disclose cosmetics, an anticipated composition claim also anticipates the intended use of such composition.

The Office Action raises the following 35 U.S.C. § 103(a) rejections:

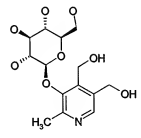
(a) Claim 4 is rejected under 35 U.S.C. § 103(a) as being unpatentable over Ogata et al. (The Journal of Vitaminology, 15, pp. 160-166 (1969)) in view of Trumbo et al.;

(b) Claim 7 is rejected under 35 U.S.C. § 103(a) as being unpatentable over Trumbo et al.; and

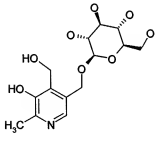
(c) Claims 9 and 10 are rejected under 35 U.S.C. § 103(a) as being unpatentable over JP 2002-265316 in combination with Trumbo et al.

The art-based rejections are respectfully traversed. In brief, Applicants respectfully submit that the compound in Trumbo identified as CAS No. 72551-78-1 was falsely identified and is in fact 5'-O-(β-D-Glucopyranosyl)pyridoxine (CAS No. 63245-12-5). The two compounds are structural isomers and differ in the position of the

glycosyl group. Trumbo discloses the compound on the right in the following figure. Applicants further submit that the compound on the right does not fall within the scope of the claims:



CAS 72551-78-1



CAS 63245-12-5

Applicants respectfully note that Trumbo discloses only one glycosylated pyridoxine derivative, which is described as 5'-O-(β-D-glucopyranosyl) pyridoxine.

In addition, Trumbo only reports that the compound was obtained through glycosylation of pyridoxine hydrochloride using seeds of alfalfa followed by purification with ion-exchange resin and gel filtration. Trumbo does not disclose analytical data to reveal the structure of the pyridoxine glycoside.

Gregory, J.F. III., who is a co-author of Trumbo, reports in J. Agric. Food Chem., vol. 35 (1987), pp. 75-82, a copy of which is provided for the Examiner's convenience, further studies of pyridoxine glycosides. In this publication, Gregory also reports the above synthesis and includes NMR data confirming the above-shown structure of CAS No. 63245-12-5. An additional publication by Gregory in Methods of Enzymology, vol. 280 (1997) (Vitamins and Coenzyme Part J), pp. 58-71, a copy of which is provided as well, show the same data.

Furthermore, Applicants respectfully submit that the Pyridoxine 5'-β-D-glucoside, i.e., the compound discussed in Trumbo is identified in CAS as No. 63245-12-5. This fact shows that CAS No. 72551-78-1 was erroneously listed in the Chemical Abstract of Trumbo and should disclose CAS No. 63245-12-5. As further evidence for the structure and the identity of compound 63245-12-5, Applicants submit herewith copies of Agric. Biol. Chem., vol. 41 (1977), pp. 1061-1067 and Bull. Res. Inst. Bioresour. Okayama Univ., vol. 5 (1980), pp. 107-120.

As a result, Applicants submit that compound CAS No. 72551-78-1 appearing under Trumbo's abstract is erroneous, but is the compound correctly identified by Gregory as CAS No. 63245-12-5.

Accordingly, the art-based rejections have no basis for anticipation or obviousness in Trumbo. The compounds of the present invention carry the glycosyl group only as R^1 in the 3-position of the pyridoxine ring, which is structurally distinguished from the compound disclosed in Trumbo. Applicants further note that the pyridoxine derivatives carrying a glycosyl group in the 3-position have advantageous properties of high photostability, a feature which, Applicants respectfully submit, is not expected by or obvious to one of ordinary skill in the art.

CONCLUSION

In view of the foregoing remarks, Applicants submit that Trumbo does not disclose a compound that falls within the scope of the present claims and that compound

CAS No. 72551-78-1 has been attributed to Trumbo in error. Withdrawal of all art-based rejections is respectfully requested.

Should there be any questions or comments, the Examiner is invited to contact the undersigned at the below-listed telephone number.

Respectfully submitted,
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Methods of Enzymology, vol. 280 (1997) (Vitamins and Coenzyme Part J), pp. 58-71;
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Identification and Quantification of Pyridoxine–β-Glucoside as a Major Form of Vitamin B_6 in Plant-Derived Foods[1]

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A high-performance liquid chromatographic procedure was adapted to the fluorometric measurement of a glucoside conjugate of vitamin B_6 compounds in foods. 5′-*O*-β-D-Glucopyranosylpyridoxine was identified by HPLC and NMR methods as the major glycosylated form of the vitamin. This conjugate, which has been shown to exhibit incomplete metabolic utilization as vitamin B_6, was found to comprise 5–70% of the total vitamin B_6 in selected fruits and vegetables. Pyridoxine–β-glucoside was not detected in animal-derived foods including meats, human milk, and cow's milk.

The existence of conjugated or "bound" forms of vitamin B_6 in various foods of plant origin has been suggested by the results of several studies. Yasumoto et al. (1977) isolated and identified 5′-*O*-β-glucopyranosylpyridoxine from rice bran. These workers evaluated the biological activity of the synthetic pyridoxine–β-glucoside (PN–glucoside) and reported that the compound was well absorbed in vitro and was well utilized as vitamin B_6 in bioassays with deficient rats (Tsuji et al., 1977). Kabir et al. (1983a) devised an indirect microbiological assay procedure for the quantitation of β-glycosylated forms as well as total vitamin B_6 in foods. The results of this assay and studies of vitamin B_6 bioavailability in human subjects suggested that β-glycosylated forms of the vitamin were not biologically available (Kabir et al., 1983b). In contrast to the results of Tsuji et al. (1977), we have recently shown that PN–glucoside is well absorbed but undergoes little metabolic utilization in rats (Ink et al., 1986).

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A high-performance liquid chromatographic method was recently developed in our laboratory that is suitable for the measurement of the biologically active B_6 vitamers in foods and other biological materials (Gregory and Feldstein, 1985). The objectives of the present study were as follows: (a) to extend this HPLC method to the quantification of the principal β-glucoside conjugate(s) of vitamin B_6 in foods; (b) to identify the principal glycosylated form(s) of vitamin B_6 in foods; (c) to determine the concentration of total and glycosylated vitamin B_6 in selected foods.

MATERIALS AND METHODS

Reagents and Samples. Pyridoxal (PL), pyridoxamine (PM), pyridoxine (PN), and 4′-deoxypyridoxine (4-dPN) were obtained as their hydrochloride salts from Sigma Chemical Co. (St. Louis, MO). Pyridoxal 5′-phosphate, 4-pyridoxic acid, and pyridoxamine 5′-phosphate also were obtained from Sigma. Water was purified for chromatographic use with a Milli-Q system (Continental Water Systems Corp., El Paso, TX). 2-Propanol (HPLC Grade) and sulfosalicylic acid (Certified ACS) were obtained from Fisher Scientific Co. (Pittsburgh, PA). 1-Octanesulfonic acid (sodium salt) was obtained from Eastman Kodak Co. (Rochester, NY). All other chemicals were of analytical grade.

Tritiated pyridoxine hydrochloride ($G\text{-}^3H$; 1.67 Ci/mmol; Amersham Corp., Arlington Heights, IL), which was found by HPLC to have a radiochemical purity of 95%, was used without further purification. The isotopic distribution reported by the manufacturer was as follows: methyl, 61.9%; 5-methylene, 2.0%; 4-methylene, 21.9%; C-6, 11.9%.

All food samples were purchased locally. Samples of human milk (approximately 30 mL of foremilk) were obtained from six local donors ranging in stage of lactation from 1–8 months. These women included three who consumed a typical mixed diet (meats, poultry, fish, fruits, cereals, vegetables, dairy products) and three who were lacto-ovo vegetarians. Several donors consumed a daily vitamin supplement providing 2 mg of pyridoxine hydrochloride.

Determination of PN–Glucoside in Foods. Extraction using sulfosalicylic acid (SSA) and purification of sample extracts by anion-exchange chromatography were performed by methods developed by Vanderslice et al. (1981) as described in our previously reported HPLC procedure (Gregory and Feldstein, 1985). Solid samples (2 g) were mixed with 9 mL of 5% (w/v) SSA and 1 mL of a solution of 4-dPN (typically 120 nmol/mL in 5% SSA) and then homogenized with a Polytron homogenizer (Brinkman Instruments Co., Westbury, NY) for 45 s at power setting 7. Ten milliliters of methylene chloride was added, and the mixture was blended for 20 s at power setting 5. The homogenates were centrifuged at 9000*g* for 15 min at 2 °C, after which an aliquot of the aqueous layer was removed.

Milk samples (3 mL) were blended in 10-mL centrifuge tubes with 1 mL of 20% (w/v) SSA and 0.15 mL of 120 nmol/mL 4-dPN with a Polytron for 45 s at power 7. The mixtures were mixed again on a Vortex mixer after the addition of 4 mL of methylene chloride for 20 s and then centrifuged.

Preparative anion-exchange chromatography was performed with a column packed with Bio-Rad AG2-X8 (Bio-Rad Laboratories, Richmond, CA), essentially as described by Vanderslice et al. (1981), to remove SSA and purify the sample extracts. The biologically active B_6 vitamers, 4-dPN, and PN-glucoside eluted as a single fluorescing peak near the void volume (retention time approximately 4 min). The eluate either was analyzed directly or was subjected to enzymatic hydrolysis prior to HPLC analysis. For enzymatic hydrolysis of PN–glucoside, 0.9 mL of the AG2-X8 eluate was diluted with 0.1 mL of 1.0 M sodium phosphate, adjusted to pH 5.0 with 1 N NaOH, and then 0.05 mL of a solution of β-glucosidase in water was added (2 mg of protein, 60 U; Sigma Chemical Co.; type I). The mixtures were incubated for 5 h in a 37 °C water bath and then deproteinated by addition of 0.07 mL of 100% (w/v) trichloroacetic acid.

The HPLC analytical system, which was identical with that described previously (Gregory and Feldstein, 1985), consisted of two solvent metering pumps (Model 110A; Altex Scientific, Berkeley, CA), a gradient controller (Altex Model 410), injection valve (altex Model 905-40 with 50-μL loop), and a fluorometric detector (Model LS-5; Perkin-Elmer Corp., Norwalk, CT) equipped with a 20-μL flow cell. An excitation wavelength of 330 nm (15-nm bandpass), an emission wavelength of 400 nm (20-nm bandpass), and a response factor of 4 were employed. An electronic integrator (Model 3388A; Hewlett-Packard, Avondale, PA) recorded and integrated chromatographic data, initiated and reset solvent programs, and controlled programmed valve switching between solvent A2 and B (described below). The analytical column was a Perkin-Elmer 3 × 3 (3 μm octadecylsilyl, 3-cm length × 4.6-mm i.d.). Fluorometric detection was enhanced by using the postcolumn phosphate–bisulfite reagent (Coburn and Mahuren, 1983), as previously described (Gregory and Feldstein, 1985). The 1.0 M potassium phosphate reagent containing 1 mg/mL sodium bisulfite (pH 7.5) was pumped into the column eluate stream at 0.2 mL/min (Model 196-31 pump; Milton-Roy Co., St. Petersburg, FL).

The following HPLC mobile phases were employed: solvent A1, 0.033 M potassium phosphate and 8 mM octanesulfonic acid (pH 2.2); solvent A2, 0.033 M potassium phosphate, 8 mM octanesulfonic acid, and 2.5% (v/v) 2-propanol (pH 2.2); solvent B, 0.033 M potassium phosphate, 6.5% 2-propanol (pH 2.2). The separation was peformed at ambient temperature by using a linear gradient (1.8 mL/min total flow rate) from 100% solvent A1 to 100% solvent A2 in 12 min, followed by a programmed switch to 100% solvent B 15 min after injection. After elution of PM, approximately 10 min were required for reequilibration prior to injection of the next sample. A blank gradient was run daily as a check for trace contamination of the water with vitamin B_6 of microbial origin. Methanol was used as the storage solvent. Quantitation was done relative to the response of the internal standard 4′-deoxypyridoxine. All phases of this procedure were performed under gold fluorescent lights, and samples were shielded with aluminum foil to minimize photochemical degradation of vitamin B_6 compounds.

Isolation of PN–Glucoside from Alfalfa Sprouts. Dry alfalfa seeds (8 g) were soaked in water overnight, then washed with water, and drained. Pyridoxine hydrochloride (25 mg) was dissolved in several milliliters of water and the resultant added to the moist seeds. The seeds were maintained in an open container in the dark at ambient temperature (ca. 22 °C) for 7 days. Water was added daily as needed to maintain hydration. After 7 days a total of 68 g of alfalfa sprouts was obtained.

For the purification of PN–glucoside, 12 g of alfalfa sprouts was minced and homogenized in 3 mL of water with a Polytron. Trichloroacetic acid was immediately added to yield a final concentration of 7% (w/v). The homogenate was centrifuged 20 min at 12000*g* at 4 °C. The

trichloroacetic acid was removed from the supernatant containing PN–glucoside by repeated extraction with diethyl ether, followed by evaporation of traces of ether under a stream of nitrogen gas.

PN–glucoside was isolated by preparative HPLC by a binary step-gradient method similar to that reported previously (Gregory and Feldstein, 1985). The preparative separation was performed on a Techsphere Ultra 5C18 column (5 μm octadecylsilyl, 5-mm i.d. × 25-cm length; HPLC Technology, Palos Verdes Estates, CA), and the following mobile phases were utilized: solvent A, 0.033 M potassium phosphate, 8 mM octanesulfonic acid, 2.5% (v/v) 2-propanol (pH 2.2); solvent B, 0.033 M potassium phosphate, 14% (v/v) 2-propanol (pH 2.2). The column was equilibrated in solvent A. After injection of 900 μL of the alfalfa extract, the column was eluted with solvent A for 5 min, followed by continued isocratic elution with solvent B for 40 min. Fractions were collected, and those containing PN–glucoside were pooled. In contrast to the analytical separation in which PN–glucoside eluted between PL and PN, PN–glucoside eluted between PMP and PL in the preparative procedure. This preparative HPLC was repeated until all of the extract had been purified.

Isolated PN–glucoside was further purified to remove the components of the HPLC mobile phase. A column (0.7-cm i.d. × 10-cm length) was packed with Bio-Rad AG50W-X8 (100–200 mesh, ammonium form) and equilibrated in 0.033 M ammonium phosphate, pH 2.2. The entire volume of pooled PN–glucoside (13 mL) was applied to the column and then washed with 10 mL of 0.05 M ammonium acetate (pH 4) followed by a convex gradient from 25 mL of 0.05 M ammonium acetate (pH 4) and 25 mL of 0.25 M ammonium acetate (pH 4). Elution was accomplished by using a convex gradient of 25 mL of 0.25 M ammonium acetate (pH 4) and 25 mL of 0.25 M ammonium acetate (pH 7). Fractions were monitored for the presence of the glucoside by HPLC. PN–glucoside eluted within the first 25 mL of the gradient. The fractions containing PN–glucoside were pooled and lyophilized. The yield of PN–glucoside was approximately 400 μg of PN equivalents (2.4 μmol) or 9% relative to the PN added (based on 12 g of sprouts). Analytical HPLC of the purified glucoside indicated the absence of PN and other B_6 vitamers.

Preparation of Tritiated PN–Glucoside in Alfalfa Sprouts. In order to evaluate the conjugation process and to determine the fate of PN–glucoside during extraction and extract purification, alfalfa sprouts (3 g of dry seeds) were grown as described above except in the presence of tritiated PN (2.0 μCi, 1.2 nmol). The tritiated PN was added after hydration of the seeds, and water was added daily as needed over the 5-day growth period. Three replicates of this procedure were performed. The total distribution of vitamin B_6 compounds was determined by extraction with SSA, purification on the AG2-X8 column, and analytical HPLC (Perkin-Elmer 3 × 3 column, ternary gradient) with fluorometric detection. The distribution of radiolabeled B_6 vitamers was determined by HPLC (Techsphere Ultra 5C18 column, binary gradient) followed by liquid scintillation spectrometry of collected fractions using Aqualyte scintillation fluid (J. T. Baker Chemical Co., Jackson, TN). Conversion of counts per minute to disintegration per minute was done by using efficiency determined from the observed H number and quench curves.

Characterization of PN–Glucoside by NMR Spectroscopy. Pooled fractions of unlabeled PN–glucoside from ion-exchange chromatography (approximately 300 μg of PN equivalents) were lyophilized to dryness. The sample was dissolved in 5 mL of deuterium oxide (99.6 atom %) and again lyophilized to achieve equilibration. The deuteriated sample was then dissolved in 1 mL of deuterium oxide containing 1 mg of sodium (trimethylsilyl)tetradeuteriopropionate (TSP; Wilmad, Buena, NJ), and the NMR spectra were recorded.

NMR spectra were obtained at 25 °C on a Nicolet NT-300 system (Nicolet Instrument Corp., Madison, WI) with a superconducting wide-bore spectrometer operated in the Fourier transform mode at 300 MHz. The 70-kG field was locked on the deuterium signal, with data acquisition in the double-precision mode due to the limited amount of sample. Spectra were recorded at a 90° angle with a sweep width of 2500 Hz.

RESULTS

Determination of PN–Glucoside. The previously reported ion-pair HPLC method for the determination of vitamin B_6 compounds in foods and other biological materials (Gregory and Feldstein, 1985) was examined to determine its suitability for the measurement of PN–glucoside. The gradient elution scheme previously described achieved separation of PN–glucoside from the other vitamers. Occasionally, a nonlinear gradient was used when improved resolution of the glucoside from PL and PN was required. PN–glucoside eluted as a fluorescing peak between PL and PN in all analyses.

Evaluation of the molar response of PN–glucoside in purified form and that present in plant extracts was performed by measuring peak areas relative to the area of the internal standard before and after enzymatic hydrolysis. These results, as illustrated in Figure 1, indicated equivalent molar fluorescence of PN–glucoside and PN. Therefore, the concentration of the glucoside may be routinely determined by measurement of the PN–glucoside peak area with quantification relative to the area of the PN standard, which eliminates the need for a purified PN–glucoside standard.

Enzymatic hydrolysis served as a convenient diagnostic tool in verifying the identity of the glucoside peak. The chromatograms of Figure 1 illustrate the effect of enzymatic hydrolysis and show representative separations. The lack of interferences was verified by the fact that, in all plant-derived samples examined, no peaks having the retention time of PN–glucoside were observed following β-glucosidase treatment.

The area of the PN–glucoside peak was linearly related to the amount injected over the entire range examined (10 pmol–100 nmol/injection). This range of linearity is consistent with that observed with other forms of vitamin B_6 in this analytical system (Gregory and Feldstein, 1985). The limit of detection (signal/noise 3) for the determination of PN–glucoside was approximately 1 pmol/50-μL injection, which corresponds to 0.06 nmol/g of sample. The linear response characteristics and high sensitivity are suitable for the measurement of PN–glucoside at levels occurring in a wide variety of foods and other biological materials.

The overall precision of the preparative and analytical chromatographic systems was evaluated through repetitive analysis of a standard mixture at levels routinely used in the assay (Table I). The observed relative standard deviation (RSD) for PN–glucoside (3.77%) was similar to that of other B_6 vitamers (2.46–5.04%).

The results of the analysis of various fruits, vegetables, and milk are shown in Table II. Analysis of replicate samples of various foods yielded RSD values for all vitamers that were generally greater than those observed for

Table I. Within-Run Precision of Preparative and Analytical Chromatographic Systems Determined by Five Replicate Analyses of a Mixed B_6 Vitamer Solution[a]

vitamer	conc, nmol/mL	RSD, %	vitamer	conc, nmol/mL	RSD, %
PLP	10	3.06	PN–glucoside	1.3	3.77
PMP	4	2.46	PN	5	5.04
PL	5	3.68	PM	2	3.69

[a] Abbreviations: RSD, relative standard deviation; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PL, pyridoxal; PN–glucoside, 5′-*O*-β-D-glucopyranosylpyridoxine; PN, pyridoxine; PM, pyridoxamine.

standards (Table II). This may reflect the added variance of extraction as well as possible heterogeneity of the samples. PN–glucoside was detected in all plant-derived samples and ranged from 5.5% of the total vitamin B_6 in bananas to 70.1% in carrots among the common foods analyzed. The complete distribution of vitamin B_6 compounds in many of these plant-derived foods has not been previously determined.

Evaluation of chromatograms (e.g., Figure 1) before and after treatment with β-glucosidase indicated that PN was the only significant glycosylated form of the vitamin in the samples analyzed. As observed in the previous study (Gregory and Feldstein, 1985), effective sample purification by the anion-exchange system (Vanderslice et al., 1980) as well as the specificity of the fluorometric detection yielded chromatograms that were largely free of non B_6 components. Of the samples analyzed to date from both plant and animal sources, the only interference found was an incompletely resolved peak that precluded measurement of PLP in peanut butter.

A peak that eluted slightly before PN–glucoside was observed in all samples of cow's milk and human milk. Repeated analysis of milk samples before and after enzymatic treatment in the presence and absence of added PN–glucoside confirmed that the pyridoxine–β-glucoside was not endogenously present in human or cow's milk (Table I). In view of the low limit of detection for PN–glucoside (1 pmol/injection), the presence of PN–glucoside at levels of 3–5% of the total vitamin B_6 in human milk could be readily detected. Enzymatic treatment of milk extracts to which exogenous PN–glucoside had been added yielded complete hydrolysis and formation of PN; however, enzymatic treatment did not alter the area or retention time of the unidentified peak in any of the milk samples.

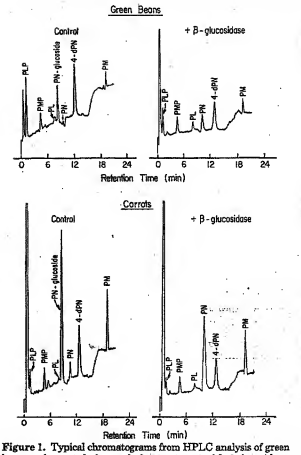

Figure 1. Typical chromatograms from HPLC analysis of green beans and carrots before and after treatment with β-glucosidase.

The HPLC data for total vitamin B_6 and the percentage of PN–glucoside were compared with previously published data concerning vitamin B_6 in these foods (Table III). General agreement between the HPLC results for total vitamin B_6 found in this study with the previously published data supports the validity of the HPLC procedure. Large differences were observed between the values for PN–glucoside percentage found in this study and the total glycosylated vitamin B_6 reported by Kabir et al. (1983a). Differences in total vitamin B_6 and percentage PN–

Table II. Concentration of PN–Glucoside and Other Forms of Vitamin B_6 in Selected Foods[a]

sample		PLP	PMP	PL	PN–glucoside	PN	PM	total B_6
raw broccoli	mean	5.82	1.65	0.85	5.62	1.91	0.15	16.0
	SD	1.32	0.38	0.18	0.84	0.71	0.04	1.8
peanut butter	mean	int	1.19	1.21	7.15	2.18	0.71	12.4
	SD		0.30	0.33	1.58	0.51	0.16	1.7
bananas	mean	3.20	0.29	0.41	1.18	12.5	4.02	21.6
	SD	0.24	0.06	0.13	0.22	1.13	0.40	1.3
raw green beans	mean	4.17	1.24	0.93	9.30	nd	0.21	15.9
	SD	0.34	0.23	0.28	0.44		0.02	0.7
raw carrots	mean	2.15	0.71	0.47	18.3	2.91	1.56	26.1
	SD	0.72	0.01	0.05	2.66	0.84	0.26	2.9
orange juice	mean	nd	1.04	0.32	5.99	1.04	0.27	8.67
	SD		0.02	0.09	0.16	0.02	0.04	0.19
cow's milk	mean	0.363	0.290	2.09	nd	nd	0.282	3.01
	SD	0.080	0.038	0.05			0.032	0.11
human milk[b]	mean	0.308	nd	0.645	nd	nd	0.039	0.992
	SD	0.038		0.062			0.001	0.112

[a] Values are given in nanomoles/gram for solids and nanomoles/milliliter for liquid foods. The orange juice was reconstituted from frozen concentrate. ND, not detected; int, PLP not measured in peanut butter due to interfering peaks. All data were derived from four independent extractions and analyses of single samples. [b] Milk sample was from a donor consuming a mixed diet and a daily 2-mg supplement of pyridoxine hydrochloride.

Table III. Comparison of HPLC Results with Previously Published Data

sample		this study	previous publications: Kabir et al.	Orr	Coburn and Mahuren
raw broccoli	total B_6, nmol/g	16.0	10.0	11.5	
	% PN-glucoside	35.1	nd (65)		
bananas	total B_6, nmol/g	21.6	18.5	30.2	
	% PN-glucoside	5.5	3		
raw green beans	total B_6, nmol/g	15.9	3.55	4.7	
	% PN-glucoside	58.5	10 (28)		
raw carrots	total B_6, nmol/g	26.1	10.1	8.9	
	% PN-glucoside	70.1	51		
orange juice	total B_6, nmol/g	8.67	2.54	2.1	
	% PN-glucoside	69.1	37		
cow's milk	total B_6, nmol/g	3.01	0.3	2.4	2.09
	% PN-glucoside	nd	nd		
human milk	total B_6, nmol/g	0.992		0.592	0.888
	% PN-glucoside	nd			

[a] Previously published data from Kabir et al. (1982a), Orr (1969), and Coburn and Mahuren (1983). The values in parentheses of Kabir are for canned products. The data of Coburn and Mahuren were obtained by HPLC analysis. Other analyses were based on microbiological growth assay.

Table IV. Concentration of Endogenous and Tritiated Vitamin B_6 Compounds in Alfalfa Sprouts

form of vitamin B_6	expt[a]	PLP	PMP	PL	PN-glucoside	PN	PM	total B_6
endogenous	1 (nmol/g)	1.95	1.81	2.37	38.9	1.96	0.86	47.9
	1 (% dist)	4.1	3.8	4.9	81.3	4.1	1.8	
tritiated	1 (% dist)				67.5	32.5		
tritiated	2 (% dist)	2.8		9.0	72.6	12.5	3.1	
tritiated	3 (% dist)	2.0	1.2	5.3	70.8	19.3	1.4	

[a] Refers to analysis of three independent preparations of alfalfa sprouts in the presence of a tracer amount of tritiated PN. % dist refers to the percentage distribution of endogenous or tritiated B-6 vitamers. Only tritiated compounds were analyzed in experiments 2 and 3.

glucoside between these studies may reflect variation in stage of maturity, varietal differences, and environmental conditions during propagation and after harvest. A direct comparison between the HPLC method and the microbiological procedure of Kabir et al. is required to resolve these questions.

The propagation of alfalfa sprouts served as a convenient means of preparing a plant tissue under controlled conditions for evaluation of B_6 vitamer distributions and as a source of PN–glucoside for further characterization. Quantification of extracted radioactivity indicated 73–97% uptake of the tritiated PN. Similar patterns of B_6 vitamer distribution indicated that the metabolism and conjugation of the labeled PN paralleled that of the endogenous vitamin B_6 (Table IV). Because the pattern of radiolabeled B_6 vitamers was determined by direct analysis of crude extracts and the endogenous B_6 vitamers were determined by HPLC following anion-exchange purification, the similarity of these results is evidence of high recovery of PN–glucoside in the preparative phase of the analytical procedure. High recovery values (>90%) were also observed for purified PN–glucoside added to food samples prior to extraction and HPLC analysis.

Identification of PN–Glucoside. The NMR spectrum of PN–glucoside dissolved in deuterium oxide is shown in Figure 2. The spectrum was obtained by using a double-precision data acquisition technique with a one-pulse experiment. Signals at δ 5.03, 5.01, 4.69, and 4.66 represent doublets of the 5′-methylene protons while the signal at δ 4.98 was derived from the 4′-methylene protons. The 5-methylene protons of underivatized (nonglycosylated) pyridoxine have been reported to elicit NMR signals at a higher field than the 4-methylene protons (Korytnyk and Paul, 1965). The signals of these methylene protons would be markedly altered by glycosylation at either position, which would be indicative of the site of the glycosidic linkage. The spectrum of PN–glucoside in this study

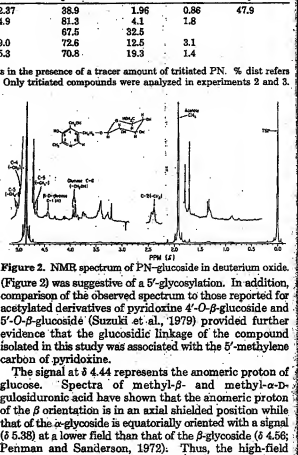

Figure 2. NMR spectrum of PN–glucoside in deuterium oxide.

(Figure 2) was suggestive of a 5′-glycosylation. In addition, comparison of the observed spectrum to those reported for acetylated derivatives of pyridoxine 4′-*O*-β-glucoside and 5′-*O*-β-glucoside (Suzuki et al., 1979) provided further evidence that the glucosidic linkage of the compound isolated in this study was associated with the 5′-methylene carbon of pyridoxine.

The signal at δ 4.44 represents the anomeric proton of glucose. Spectra of methyl-β- and methyl-α-D-gulosiduronic acid have shown that the anomeric proton of the β orientation is in an axial shielded position while that of the α-glycoside is equatorially oriented with a signal (δ 5.38) at a lower field than that of the β-glycoside (δ 4.56; Penman and Sanderson, 1972). Thus, the high-field position (δ 4.44) of the anomeric proton in this spectrum indicates a β-glucosidic linkage. The observed coupling constant (approximately 6 Hz) of the δ 4.44 signal also is evidence of a trans-diaxial arrangement of the anomeric proton and the proton of the adjacent (C-2) carbon, which is similar to the spectrum of methyl-β-D-gulosiduronic acid (Penman and Sanderson, 1972). The signal at δ 2.38 is derived from the methyl protons at the 2-position of PN. Signals found at δ 3.2–3.8 are associated with hydroxy-

methyl and other protons of glucose. The sharp signals at δ 1.92 and 0 were due to residual acetate from the isolation procedure and the TSP reference, respectively.

DISCUSSION

The results of the studies reported here indicate that 5′-*O*-β-D-glucopyranosylpyridoxine (PN–glucoside) represents a substantial proportion of the vitamin B_6 in many fruits and vegetables. The identity of this conjugated form of vitamin B_6 was verified by (a) conversion of the glucoside to PN by treatment with β-glucosidase and (b) NMR spectra indicating conjugation to the 5′-position of PN by an *O*-β-glycosidic linkage.

These studies have established the applicability of reversed-phase ion-pair HPLC to the determination of PN–glucoside in foods. As shown in this study and in previous research (Gregory and Feldstein, 1985), this HPLC method permits an accurate and highly sensitive determination of vitamin B_6 compounds in foods and biological materials. Factors contributing to the validity of the method include an effective sample extraction and ion-exchange purification procedure (Vanderslice et al., 1980), high-efficiency chromatographic HPLC separation, sensitive fluorometric detection facilitated by postcolumn addition of strongly buffered bisulfite solution (Coburn and Mahuren, 1983), and individual quantification of vitamin B_6 compounds using 4′-deoxypyridoxine as an internal standard. The precision, sensitivity, and recovery observed for PN–glucoside observed here were comparable to those of other vitamin B_6 compounds.

With the exception of bananas, in which the PN–glucoside exists as a minor constituent, the plant-derived samples examined contained approximately 35–80% of their vitamin B_6 as PN–glucoside (Tables III and IV). PN–glucoside was not detected in liver or muscle samples from rats, cattle, or swine. Recent studies in our laboratory concerning the metabolism of PN–glucoside have indicated that β-glucosylation of B_6 vitamers does not occur in vivo in the rat (Ink et al., 1986), and no evidence of the formation of other conjugated forms of the vitamin has been seen. The absence of PN–glucoside from milk suggests that the primary route of elimination of dietary PN–glucoside from the body is by urinary excretion, as recently reported (Ink et al., 1986), with little or no secretion by the lactating mammary gland in the human and the cow. It was particularly noteworthy that PN–glucoside was not detected in any of the human milk samples, regardless of the diet of the donor (mixed vs. lacto-ovo vegetarian). The data for vitamin B_6 compounds in human milk were from replicate analysis of a single sample (Tables II and III). These data (total concentration and distribution of B_6 vitamers) were similar to results from analysis of samples from other donors.

The measurement of glycosylated forms of vitamin B_6 has been done previously by differential microbiological assay after hydrolytic treatments (Kabir et al., 1983a; Yasumato et al., 1976). Kabir et al. (1983a) examined various fruits and vegetables and reported results for β-glycosylated vitamin B_6 that differed somewhat from those found in this study. Underestimation of glycosylated vitamin B_6 in samples containing endogenous β-glucosidase activity may have been a limitation of their assay, as suggested by the data of Table III. The use of sulfosalicylic acid as an extractant in this study rapidly inactivates hydrolytic enzymes and, thus, minimizes these losses. Yasumoto et al. (1976) also utilized a microbiological assay procedure for the examination of "bound" forms of vitamin B_6 in various cereal grains and seeds. Unidentified forms of the vitamin that became active for the assay organism following β-glucosidase treatment were detected in all samples analyzed in that study. Ion-exchange chromatographic analysis of enzymatic hydrolysis products indicated that PN was the major form of vitamin B_6 released by β-glucosidase treatment (Kabir et al., 1983a; Yasumato et al., 1976). HPLC analysis of enzymatically hydrolyzed samples in this study (Figure 1) directly confirmed these observations. Similar results were also reported by Nelson et al. (1977) concerning the analysis of an unidentified bound form of vitamin B_6 in orange juice.

The existence of two minor esterified derivatives of PN–glucoside pea seedlings has been recently reported. Tadera et al. (1983) found a PN–glucoside in which the C-6 position of the glucopyranosyl moisty was esterified to 3-hydroxy-3-methyl-4-carboxybutanoic acid, while in later studies they found the analogous malonyl ester (Tadera et al., 1985). Further research is needed to determine the quantitative significance of these esterified forms of PN–glucoside in foods, although the results of the present study suggest that such derivatives of PN–glucoside are negligible in common plant-derived foods. Research has shown that a glucosyltransferase of pea seedlings exhibits high specificity for uridinediphospho-(UDP)glucose as the glucose donor in the conjugation of PN in vitro (Tadera, 1982). The source of the acyl moiety in the esterified derivatives is unclear at this time.

A major implication of this research involves the nutritional properties of PN–glucoside. Research employing radiolabeled PN–glucoside in rats indicated little in vivo utilization in vitamin B_6 metabolism (Ink et al., 1986). The urinary clearance of PN–glucoside was nearly 4 times faster than for nonglycosylated vitamin B_6, while intact PN–glucoside represented 85% of urinary labeled vitamin B_6 compounds. Low apparent bioavailability also was reported by Kabir et al. (1983b) in studies of the utilization by human subjects of vitamin B_6 from several foods. Most information concerning the content of vitamin B_6 in foods has been derived by microbiological growth assays using vitamin B_6 dependent yeasts (Toepfer and Polansky, 1970). The procedure recommended for samples of plant origin require heating of the sample in HCl at 121 °C in 0.44 M HCl for 2 h (AOAC, 1970), which induces complete hydrolysis of PN–glucoside as well as phosphorylated forms of the vitamin. Thus, microbiological assay procedures such as these would overestimate the biologically available vitamin B_6 in foods. The extent of hydrolysis as influenced by other combinations of time, temperature, and acid concentration have not been examined. Earlier microbiological assays based on extractions with dilute sulfuric acid (Atkin et al., 1943) presumably would yield at least partial hydrolysis, although this has not been determined. The HPLC method reported here, which utilizes much milder extraction conditions and permits a direct measurement of PN–glucoside, would yield data that could be interpreted with regard to the low bioavailability of PN–glucoside.

The high concentration of PN–glucoside in many plant-derived foods suggests a widespread incomplete bioavailability of the vitamin in human diets, which could not be evaluated accurately by current food composition tables. The absence of PN–glucoside from meats and milk is further evidence of high bioavailability of the vitamin in these foods. The potential existence of PN–glucoside or a derivative in human milk from women ingesting large proportions of their vitamin B_6 in the conjugated form requires further study.

In summary, 5′-*O*-β-D-glucopyranosylpyridoxine has been found to be a common component of the vitamin B_6

of plant-derived foods. The HPLC procedure reported here permits a direct assessment of the concentration of this compound and represents the first instrumental method that provides results that would be relevant to the bioavailability of vitamin B_6 in foods.

ACKNOWLEDGMENT

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Application of in Vitro Methods To Assess the Nutritive Value of Leaf Protein Concentrates

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Sixteen leaf protein concentrates (LPC) were prepared from different crops by different processes and either freeze dried or oven dried in the 1983–1985 seasons. Total lysine and chemically available lysine of these samples were estimated. Biological assay parameters such as N retention and apparent digestibility were evaluated. Total lysine and chemically available lysine show good correlation with results from biological assay. Predicted biological nutritive values show good agreement with those from in vitro studies.

Processing conditions affect the nutritive value of food proteins. Rat assays are the best methods of nutritive value evaluation. Some in vitro methods such as digestibility with proteolytic enzymes (Buchnan, 1969; Saunders et al., 1973), growth of tetrahymena (Lezander et al., 1970; Smith and Pena, 1977), and microbial availability of essential amino acids (Henry and Ford, 1965) were used for nutritive evaluation of LPC. Shurpalekar et al. (1966) and Bickoff et al. (1975) using protein efficiency ratio assays with rats found considerable losses of nutritive value on thermal drying of LPC curd as compared with freeze drying. Henry (1964), who determined the biological value and true digestibility of LPC samples, found that hot-air drying in particular reduced the true digestibility of LPC. Byers (1971) found that damage to the LPC occurred during the heat coagulation stage, particularly to its lysine content. Proteins with lysine as a first limiting amino acid might, therefore, be subject to more severe reductions in nutritive value than would those deficient in methionine (Knipfel et al., 1975).

Biological nutritive evaluation methods are costly and time consuming. Hence, attempts are made to find quicker and simple chemical methods to predict the biological nutritive value parameters. In LPC preparations methionine is usually the first limiting amino acid. In some LPC preparations, due to low availability lysine could be the second deficient or limiting amino acid (Ohshima, 1985). Thus, digestibility and availability of methionine and lysine are the chief factors that govern the nutritive value. Processing conditions affect both sulfur amino acids and lysine in the same way. Since methods for determination of available lysine have worked out well for studies on factors affecting the nutritive value of LPC, a mathematical relation between available lysine and in vivo rat assay parameters such as apparent digestibility and N retention will be highly useful.

In order to evolve a suitable mathematical relationship between chemically available lysine and apparent diges-

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Analysis of Product

The pyridoxyl peptide–oligonucleotide conjugate had characteristic absorbance peaks for the oligonucleotide and pyridoxyl groups. The pyridoxyl peptide–oligonucleotide conjugate was compared with the oligonucleotide and peptide–oligonucleotide conjugate by polyacrylamide gel electrophoresis (Fig. 2). Samples were run on a nondenaturing 20% (w/v) polyacrylamide gel in a buffer of 40 *m*M morpholinepropanesulfonic acid (MOPS) and 10 *m*M sodium acetate, adjusted to pH 7.0 with sodium hydroxide. The samples were suspended in 20 μl of loading buffer [99% (v/v) formamide], heated to 90° for 2 min, chilled on ice, and loaded on the gel. In comparison to the amino-link oligodeoxynucleotide, the peptide–oligonucleotide conjugate was severely retarded in mobility. The pyridoxyl peptide–oligonucleotide conjugate migrated slightly faster than the peptide–oligonucleotide conjugate.

[7] Preparation of Nonlabeled, Tritiated, and Deuterated Pyridoxine 5′-β-D-Glucoside and Assay of Pyridoxine-5′-β-D-glucoside Hydrolase

By JESSE F. GREGORY III and HIDEKO NAKANO

Pyridoxine 5′-β-D-glucoside exists as a major form of vitamin B_6 in many fruits, vegetables, and cereal grains and, thus, comprises a major form of dietary vitamin B_6.[1] Although several other naturally occurring forms of vitamin B_6 glucosides also have been identified, pyridoxine 5′-β-D-glucoside is the primary glycosylated form of vitamin B_6 in human diets. Metabolic utilization of the pyridoxyl moiety cannot occur without prior hydrolysis of the glycosidic bond. Almost no glycosylated pyridoxamine or pyridoxal exists naturally. Little or no glycosylated vitamin B_6 exists in animal-derived foods. Isotopic studies with rats have shown that orally administered pyridoxine 5′-β-D-glucoside either is absorbed in intact form or undergoes enzymatic hydrolysis, apparently in the small intestine.[1–3] Pyridoxine 5′-β-D-glucoside in rats exhibits approximately 25% of the vitamin B_6 activity as free pyridoxine, which reflects the incomplete hydrolysis of the glucosidic bond.[2,3] In contrast, orally administered pyridoxine 5′-β-D-glucoside in humans exhibits approximately 50% of the vitamin B_6 activity as pyridoxine.[4]

Nutritional and biochemical studies and instrumental measurements of pyridoxine 5′-β-D-glucoside are complicated by the lack of commercial sources of this compound. Chemical synthesis has been reported in which 3-*O*-isopropylidine-pyridoxine is reacted with 2,3,4,6-tetra-*O*-acetyl-α-D-glucosyl bromide to yield, following removal of protecting groups, pyridoxine 5′-β-D-glucoside primarily as the β anomer.[5] A variety of higher plants can produce pyridoxine β-D-glucosides (at the 4′ or 5′ position, or both) during germination in the presence of exogenous pyridoxine, as do several microorganisms.[6–8] We have examined the biological synthesis based on the germination of peas and alfalfa for preparation of pyridoxine 5′-β-D-glucoside and found alfalfa to be preferable in yield and convenience. The preparative methods reported here involve biological syntheses using germinating alfalfa seeds.[2,9] We believe that these methods are advantageous in comparison to other potential methods of biosynthesis or chemical synthesis in terms of simplicity, convenience, and suitability for the preparation of nonlabeled, tritiated, and deuterated forms of pyridoxine 5′-β-D-glucoside. These synthetic methods and purifications described provide various forms of pyridoxine 5′-β-D-glucoside in high purity and sufficient quantity for use in nutritional and biochemical studies.

The partial availability of pyridoxine 5′-β-D-glucoside as a source of vitamin B_6 for mammals provides evidence of the existence of enzyme activity capable of hydrolyzing the β-glycosidic bond.[2,4,10] More direct evidence of such activity has been observed in a study of rat intestine (using pyridoxine 4′-β-D-glucoside as substrate).[11] Supernatant fractions of mammalian jejunal mucosa exhibit the ability to hydrolyze pyridoxine 5′-β-D-glucoside.[12] In rats, jejunal pyridoxine-5′-β-D-glucoside hydrolase activity increases during vitamin B_6 deficiency.[13] The microflora of rat jejunum

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also exhibit pyridoxine-5′-β-D-glucoside hydrolase activity, although this decreases during vitamin B_6 deficiency. Kidney is another significant source of pyridoxine-5′-β-D-glucoside hydrolase activity in rats. We have found that pyridoxine-5′-β-D-glucoside hydrolase[14] is a distinct protein and differs markedly from the previously identified cytosolic broad-specificity β-glucosidase.[15] In addition to methods for synthesis of pyridoxine 5′-β-D-glucoside, we report here an improved method for assay of pyridoxine-5′-β-D-glucoside hydrolase activity.

Preparation of Various Forms of Pyridoxine 5′-β-D-Glucoside

Materials

Fresh alfalfa seeds suitable for sprouting are used in this procedure and are available at many fresh produce markets. The methods reported here permit the preparation of pyridoxine 5′-β-D-glucoside in nonlabeled, deuterated, or tritiated form. Nonlabeled pyridoxine hydrochloride (Sigma Chemical Co., St. Louis, MO), [5′-$^2H_2$]pyridoxine hydrochloride (prepared according to Coburn *et al.*),[16] and [^{3}H]pyridoxine hydrochloride (Amersham Corporation, Arlington Heights, IL; 1.4 Ci/mmol), respectively, are suitable for this synthesis. [^{3}H]Pyridoxine hydrochloride is purified if needed by reversed-phase high-performance liquid chromatography (HPLC) using a commercial octadecylsilyl column (e.g., Microsorb-MV C_{18}, 3 μm, 4.6 mm i.d. × 15 cm long; Rainin Instrument Co., Woburn, MA) with either ultraviolet (UV) absorption (280 nm) or fluorescence (excitation, 295 nm; emission, 405 nm) detection and a volatile isocratic mobile phase [0.1 *M* formic acid and ~2% (v/v) acetonitrile, adjusted as needed]. The manually collected pyridoxine peak is evaporated to dryness under a stream of nitrogen gas prior to use. Nonlabeled and deuterated pyridoxine are normally obtained in high purity and may be used without further purification.

Methods

Nonlabeled Pyridoxine 5′-β-D-Glucoside. This biological synthesis is similar to that first reported by our laboratory,[2,9] while the purification is a modification of the method of Trumbo *et al.*[2] Pyridoxine hydrochloride (~60 mg) is dissolved in 25 ml of water and used for addition to the alfalfa, as follows. Twenty grams of alfalfa seeds are soaked in water overnight in a 1-liter beaker at ambient temperature, the excess water poured off through cheesecloth, and then ~12 mg of pyridoxine hydrochloride is added in 5 ml of water and stirred briefly to disperse. Depending on the concentration of the pyridoxine hydrochloride and the acidity of the water in preparations such as this, neutralization of the pyridoxine solution with sodium bicarbonate before addition may enhance the germination and growth. The seeds are kept at ambient temperature in the dark for ~5 days. Each day ~20 ml of water is added, gently agitated, decanted, and 5 ml of the pyridoxine stock solution added. This washing and pyridoxine addition are conducted daily, except on day 5. Additional water is added as needed daily to keep the seeds and sprouts moist. Daily addition of pyridoxine as described here seems to give better growth than obtained with one single addition of ~60 mg of pyridoxine, as used initially. The total yield of sprouts (~150 g) is washed gently with water to remove excess pyridoxine, then homogenized with 150 ml of 14% (w/v) trichloroacetic acid, followed by centrifugation for 20 min at 19,000 *g* at 4°. To the resulting supernatant, acetic acid is added to reach a final concentration of 0.01 *M*, followed by adjustment to pH 4.5 with sodium hydroxide.

Pyridoxine 5′-β-D-glucoside is purified by sequential cation-exchange and gel-filtration chromatography. The entire pH 4.5 solution is applied to a column (2.5 × 30 cm) packed with AG 50W-X8 (100–200 mesh, NH_4^+ form), previously equilibrated with 0.05 *M* ammonium acetate, pH 4.5. After applying the sample, the column is washed with 200 ml of 0.05 *M* ammonium acetate, pH 4.5, then elution is accomplished with a linear gradient composed of 1 liter of this buffer and 1 liter of 0.15 *M* ammonium acetate, pH 7.0. The absorbance of collected fractions at 325 nm is determined to identify those that contain pyridoxine 5′-β-D-glucoside. If needed, HPLC is used as described below to identify fractions containing pyridoxine 5′-β-D-glucoside. The collected fractions containing this product are pooled, lyophilized, and dissolved in a minimal amount of water.

Gel-filtration chromatography removes most residual pyridoxine and other small molecules, and aids in further desalting. Single portions of ~10 ml each are individually applied and eluted from a column packed with Sephadex G-10 (2.5 × ~30 cm; previously equilibrated with water). The column is eluted with distilled water, and fractions are monitored and the product lyophilized, as described previously.

Final desalting, if needed, is achieved by dialysis (*M*, 100 cutoff cellulose ester membrane; Spectra/Por, Laguna Hills, CA) against several changes of distilled water at 4°. The retentate, which contains the pyridoxine 5′-β-

[14] H. Nakano, M. D. Levy, and J. F. Gregory, *FASEB J.* **9**, A986 (1995) [Abstract 5727].
[15] R. H. Glew, V. Gopalan, G. W. Forsyth, and D. J. VanderJagt, *in* "β-Glucosidases: Biochemistry and Molecular Biology" (A. Esen, ed.), pp. 83–112. American Chemical Society, Washington, DC, 1993.
[16] S. P. Coburn, C. C. Lin, W. E. Schaltenbrand, and J. D. Mahuren, *J. Labeled Compounds Radiopharm.* **19**, 703 (1982).

D-glucoside, is then lyophilized. It may then be reconstituted to the desired concentration and stored in convenient vials for use as needed.

Although pyridoxine 5′-β-D-glucoside typically comprises ~75% of the total vitamin B_6 in the alfalfa sprouts, yields of this procedure are low (often 35–60%) owing to losses in handling and chromatography. The product exhibits high stability when stored for up to several days in neutral solution at 4°. Little or no hydrolysis occurs during frozen storage for months. Because the UV spectrum of pyridoxine 5′-β-D-glucoside is almost equivalent to that of pyridoxine, the concentration of stock solutions can be determined using published absorptivity values (pH 7.0: 254 nm, 3760 M^{-1} cm^{-1}; 324 nm, 7100 M^{-1} cm^{-1}).[17] The ^{1}H nuclear magnetic resonance (NMR) spectrum of this product confirms that the glucosyl moiety is linked to the 5′ position of pyridoxine via a β-glycosidic bond.[9]

Deuterium-Labeled Pyridoxine 5′-β-D-Glucoside. Deuterium-labeled pyridoxine can be prepared as the [$^2H_5$], [$^2H_3$], or [$^2H_2$] forms (also termed [d5], [d3], and [d2] forms in Ref. 16). We have prepared [5′-$^2H_2$]pyridoxine 5′-β-D-glucoside, rather than other isotopomers, because of the relative convenience of synthesizing the [5′-$^2H_2$]pyridoxine starting material using the method of Coburn *et al.*[16] The biological synthesis is conducted as described previously. In a typical preparation, we use 12 g of alfalfa seeds germinated in the presence of 62 mg (297 μmol) of [$^2H_2$]pyridoxine hydrochloride, followed by cation exchange and gel filtration. The yield of [$^2H_2$]pyridoxine 5′-β-D-glucoside is 139 μmol (47%). Because of the large quantity of [$^2H_2$]pyridoxine used, there is no difference between the isotopic enrichment of the [$^2H_2$]pyridoxine starting material and the product, which indicates that isotopic dilution by endogenously synthesized pyridoxine 5′-β-D-glucoside is not significant.

Tritium-Labeled Pyridoxine 5′-β-D-Glucoside. [^{3}H]Pyridoxine (300 μCi) is added to 10 g of alfalfa sprouts essentially as previously described.[2,9,10] In typical preparations using this method, [^{3}H]pyridoxine 5′-β-D-glucoside comprises approximately 75% of the total radioactive vitamin B_6 in the sprouts. The recovery following is similar to that of nonlabeled and deuterated forms. Substantial isotopic dilution occurs from endogenous vitamin B_6 synthesis, as indicated by the specific radioactivity of ~59 mCi/mmol.

High-Performance Liquid Chromatography Analysis of Pyridoxine 5′-β-D-Glucoside and Other Forms of Vitamin B_6

The purity of pyridoxine 5′-β-D-glucoside preparations can be readily determined by ion-pair reversed-phase HPLC, as described below.[18] Analysis of vitamin B_6 compounds in other biological materials (e.g., many foods, animal tissues, and urine) also can be performed using this method by incorporation of extraction using *meta*-phosphoric acid.[18,19] Standards are obtained commercially (e.g., Sigma Chemical Co.; pyridoxine hydrochloride, pyridoxal hydrochloride, pyridoxamine dihydrochloride, pyridoxal phosphate, pyridoxamine phosphate, and the internal standard 4-deoxypyridoxine).

The HPLC is performed using a binary gradient system with one pump (i.e., pump B) adapted for switching between either of two mobile phases at the inlet. The column (Microsorb-MV C_{18}, 3 μm, 4.6 mm i.d. × 15 cm long; Rainin Instrument Co.) is equilibrated in buffer A1 (0.033 *M* phosphoric acid, pH 2.2, containing 8 m*M* octanesulfonic acid). Buffer A2 consists of 0.033 *M* phosphoric acid, pH 2.2, containing 8 m*M* octanesulfonic acid and 5% (v/v) 2-propanol, while buffer B is 0.033 *M* phosphoric acid, pH 2.2, and 8% (v/v) 2-propanol. The elution program (1 ml/min) consists of (1) a linear gradient from 100% buffer A1 to 100% buffer A2 in 4 min, (2) holding at 100% buffer A2 for 3 min, and (3) switching to 100% buffer B and maintain for 10 min (total run time, 26 min). At this point, the valve is switched from solvent B back to A2, and a linear gradient from A2 to A1 is run in 4 min, followed by 15 min of 100% A1 for reequilibration prior to the next injection. This gradient is easily automated using commercially available HPLC equipment. Detection of the vitamin B_6 compounds is accomplished by monitoring their native fluorescence at 405 nm using excitation at 295 nm. Pyridoxine and pyridoxine 5′-β-D-glucoside exhibit equivalent fluorescence response, although they are readily separated (Fig. 1).

[17] S. A. Harris, E. E. Harris, and R. W. Burg, *in* "Kirk-Othmer Encyclopedia of Chemical Technology" (H. F. Mark, J. J. McKetta, and D. F. Othmer, eds.), 2nd Ed., Vol. 16, p. 806. John Wiley & Sons, New York, 1968.

[18] H. Nakano and J. F. Gregory, *J. Nutr.* 125, 926 (1995).

Assay of Pyridoxine-5′-β-D-glucoside Hydrolase

Principle

Molecular characterization and clarification of the function of pyridoxine-5′-β-D-glucoside hydrolase requires a convenient assay procedure. Initial assays of pyridoxine-5′-β-D-glucoside hydrolase activity were based on incubations with [^{3}H]pyridoxine 5′-β-D-glucoside, followed by HPLC, collection of fractions, and scintillation counting of pyridoxine and pyridoxine 5′-β-D-glucoside peaks.[12] Because of low reaction rates and rather low specific radioactivity of the substrate, the assay was cumbersome and imprecise. We have modified this assay by using nonlabeled pyridoxine 5′-β-D-glucoside as substrate in shorter incubations. Quantification is based on direct fluorometric measurement of the product (pyridoxine) in a rapid,

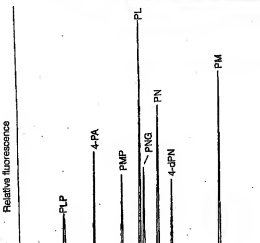

FIG. 1. Typical separation of vitamin B_6 compounds by ion-pair reversed-phase HPLC. PLP, Pyridoxal phosphate; 4-PA, 4-pyridoxic acid; PMP, pyridoxamine phosphate; PN, pyridoxine; PNG, pyridoxine 5′-β-D-glucoside; PL, pyridoxal; 4-dPN, 4-deoxypyridoxine; PM, pyridoxamine.

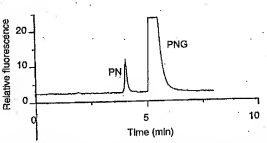

FIG. 2. Typical HPLC separation of pyridoxine (PN) and pyridoxine 5′-β-D-glucoside (PNG) in an assay of pyridoxine-5′-β-D-glucoside hydrolase activity.

isocratic HPLC procedure that is readily automated.[18] These changes improve sensitivity, precision, and speed.

Method

The assay of pyridoxine-5′-β-D-glucoside hydrolase is conducted as reported by Nakano and Gregory.[18] Tissue samples are homogenized (Polytron; Brinkmann Instruments, Inc., Westbury, NY) in 3–10 vol of 10 m*M* sodium phosphate, pH 7.0, containing 10 m*M* 2-mercaptoethanol and 1 m*M* phenylmethylsulfonyl fluoride, then centrifuged at 20,000 *g* for 20 min at 4°. The supernatant is adjusted with acetic acid to pH 6.0, followed by centrifugation at 20,000 *g* for 20 min at 4°. This supernatant is used for activity assays.

Assays are typically conducted with pyridoxine 5′-β-D-glucoside as substrate at a final dilution of 0.25 m*M* in a 40 m*M* sodium acetate buffer, pH 6.0. Incubations are at 37° for 60 min. Reactions are stopped by incubation in a boiling water bath for 3 min. The concentration of pyridoxine produced is determined by reversed-phase HPLC using an isocratic mobile phase [0.033 *M* phosphoric acid, pH 2.2, containing 0.25% (v/v) 2-propanol] and an octadecyisilyl column (Microsorb-MV C_{18}, 3 μm, 4.6 mm i.d. × 15 cm long; Rainin Instrument Co.) and measurement of native fluorescence (excitation, 295 nm; emission, 405 nm) (Fig. 2). A refrigerated autosampler permits analysis of many assay mixtures with injections every 10 min.

Appropriate blanks must be analyzed to assess whether any pyridoxine is present in the substrate as a contaminant or through nonenzymatic hydrolysis from prolonged storage. It should be noted that the apparent K_m for pyridoxine-5′-β-D-glucoside hydrolase is ~1.5 m*M*.[12,14] Thus, the concentration of substrate needed to approximate zero-order kinetics would be ~20 m*M*. Routine assays at this high substrate concentration are prohibitive because of the quantity of pyridoxine 5′-β-D-glucoside needed. To conserve substrate, our routine assays are generally conducted at ≤1 m*M* pyridoxine 5′-β-D-glucoside. The resulting kinetic differences should be recognized when using this procedure.

Acknowledgment

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Isolation from Rice Bran of a Bound Form of Vitamin B_6 and Its Identification as 5′-O-(β-D-Glucopyranosyl) Pyridoxine

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Received February 4, 1977

One of the bound forms of vitamin B_6 occurring in rice bran was isolated in a faintly yellowish syrup by repeating ion-exchange and paper-partition chromatographic techniques. The behaviors of the isolate on thin-layer and Aminex A-5 column chromatograms were coincident with those of synthetic pyridoxine-β-D-glucoside which was obtained by Königs-Knorr condensation of α^4,3-O-isopropylidene pyridoxine and 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide. On acid hydrolysis, the isolate gave pyridoxine and glucose. Glucose was proved to bind to the 5-hydroxymethyl group of pyridoxine, because the isolate did not react with 2,6-dichloroquinone chlorimide in the presence of boric acid. An equimolar amount of pyridoxine and D-glucose was produced with an equivalent consumption of the isolate by the action of β-glucosidase. No essential difference between the isolated and synthetic preparations could be detected in UV- and NMR-spectral features. Thus, the chemical structure of the isolate was identified as 5′-O-(β-D-glucopyranosyl) pyridoxine.

Description of vitamin B_6 nutrition has been considerably obscured by the two obstructions, the multiplicity of its function in metabolism and the complexity of its forms occurring in foods-especially in whole grain cereals. In view of the multiplicity, it is not surprising that the human dietary requirement for the vitamin has not yet been definitely established. The factors influencing the requirement have not yet been systematically studied and experimental data so far presented are fragmentary and some are conflicting or of wide diversity; estimates of requirements during pregnancy range from "the level of 2.5 mg[1] to 15 and 20 mg per day,[2] for example.

In view of the complexity, it is not unexpected that the studies on the exact quantitation of the dietary vitamin B_6 actually available for nutrition has been beset by methodological difficulties. Free or easily extractable vitamin B_6 occurring in food may be measured by homogenization and subsequent microbiological assay or chemical determination, such as described by the present authors.[3,4] However, a large part of vitamin B_6 present in food is in bound forms that behave differently from those of free vitamin. This is especially true with whole grain cereals. For example, rice bran extracts, when fractionated by gel-filtration, gave a variety of PIN-containing compounds which were unavailable to the assay microorganism unless hydrolysed.[3] It is thus apparent that the availability of these compounds as vitamin B_6 is very limited, owing to the limited capabilities of the organisms employed for the assay to convert the bound forms to the free active form, so as to open an important problem which must be resolved if reasonably accurate estimates of the vitamin requirements or the adequacy of diets with regards to their B_6 vitamers content can be evaluated. The nature of these

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Abbreviation: PIN, pyridoxine; PAL, pyridoxal; PAM, pyridoxamine; PIN-β-G, 5′-O-(β-D-glucopyranosyl)pyridoxine; PIC-α-G, 5′-O-(α-D-glucopyranosyl)-4-pyridoxic acid; PIN-α-G, 5′-O-(α-D-glucopyranosyl) pyridoxine.

compounds and their availability to mammalian nutrition have been occupying the attention of the present authors.

The present paper describes isolation and identification of the PIN derivative that is inferred to occur in rice bran from the previous finding of the present authors: a considerable part of the bound vitamin B_6 gives free PIN after prolonged incubation with either β-glucosidase or takadiastase.[8]

MATERIALS AND METHODS

Materials. Defatted rice bran was kindly supplied by Ogawa Oil Manufacturing Co. (Nagaokakyo City, Kyoto). A vitamin B_6-depleted basal medium for microbiological assay with *Saccharomyces carlsbergensis* 4228 was obtained from Nissui Pharm. Chemical Co. α-Glucosidase (baker's yeast), β-glucosidase (sweet almond), glucose oxidase (*Aspergillus niger*), and peroxidase (horseradish) were purchased from Sigma Chemical Co. Other chemicals were of the highest grade commercially available.

Determination of pyridoxine and its vitamers on semi-automated analyser system or by microbiological assay. Analyses of free and bound forms of vitamin B_6 on the semi-automated analyser system were conducted as described previously.[9] Microbiological assay of the vitamin was carried out on the samples of before and after hydrolysis (0.4 N HCl, 130°C, 1 hr), according to the method of Atkin *et al.*[10] as modified by Fukui *et al.*,[11] utilizing *Saccharomyces carlsbergensis* 4228 as test organism. The difference in the vitamin content of these two assays gives a measure for the content of bound forms of the vitamin in the sample.

Hydrolysis by α- or β-glucosidase. The isolated compound was incubated at 37°C with 1.2 or 3 units of α- or β-glucosidase in 2.5 ml of 0.1 M (pH 6.8) phosphate buffer or 0.2 M (pH 4.8) acetate buffer, respectively. A unit of glucosidase was defined as the amount of enzyme capable of releasing one μmole of *p*-nitrophenol per min from 10 mM *p*-nitrophenyl-α- or -β-glucoside under the above described conditions. An aliquot of the reaction mixture (0.2 ml) withdrawn at appropriate intervals was added to 1 ml of distilled water. The sample was placed immediately, and kept for 2 min, in a boiling water bath to inactivate the enzyme, and the amount of D-glucose released was determined by the method of Lloyd and Wholem[6] using glucose oxidase-peroxidase system.

Spectrometry. UV and NMR spectra were taken on the same instruments under the same conditions as described previously.[9]

Chemical synthesis of PIN-β-G. PIN-β-G was obtained by Königs-Knorr condensation of α',3-O-isopropylidene pyridoxine (I) and 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (II) and subsequent removal of protecting groups. Compounds I and II were synthesized according to the procedures described by Kocyntnyk and Wiedeman[10] and Bates,[12] respectively. A mixture of compound I (2.5 g), II (5.0 g) and silver carbonate (7.0 g) in dry ether was allowed to stand for 6 days in the dark with constant stirring. After removal of insoluble materials, the supernatant was applied to a Wakogel C-100 column (2.0φ×45 cm), followed by elution successively with benzene (200 ml), benzene-ether (5: 1, 200 ml) and ethylacetate. Active fractions giving positive reactions both with anthrone-sulfuric acid[13] and with diazotized 5-chloroanilide reagent[3] on thin-layer chromatogram, after detachment of the phenolic OH-protecting group, were combined and evaporated to dryness *in vacuo*. An ethereal solution of the resultant product was again applied to the same Wakogel column and then eluted with ether-ethylacetate (4: 1). The active fractions of the effluent were chromatographed on an activated Alumina 300 column (1.2φ×17 cm) by elution with ether (60 ml) and subsequently with ethylacetate (70 ml). β-Anomer of tetraacetyl-D-glucopyranosyl isopropylidene pyridoxine was mostly found in the latter effluent, concomitantly with small amounts of α-anomer. The most active fractions, after being evaporated to dryness, were dissolved in a solution of ethanol-1% formic acid (3: 21) and refluxed in a boiling water bath for 90min; thereby, the isopropylidene group was completely eliminated. The residual syrup after *in vacuo* evaporation was dissolved in 1 N NaOH and kept at 35°C for 40 hr to remove the acetyl groups from the glucose moiety. Thus, 5'-O-(β-D-glucopyranosyl)pyridoxine was recovered from hot alcohol as white powders; yield, 100 mg (mp 169~174°C). *Anal.* Found: C, 50.59; H, 6.27; N, 4.27. Caled. for $C_{14}H_{23}NO_8$: C, 50.76; H, 6.34; N, 4.23%. Its chemical structure was substantially ascertained by UV and NMR analyses and chromatographic behaviors before or after α- or β-glucosidase hydrolysis.

RESULTS

Isolation of a bound vitamin B_6

The respective fractions in the following isolation procedure were assayed for bound vitamin B_6 by the differential microbiological method as described in MATERIALS AND METHODS. Defatted rice bran (about 20 kg) was suspended in 4 volumes of distilled water

with adjustment to pH 3.7 by the addition of dilute HCl. The suspension was gently stirred for 4 hr at 40°C and then strained through a cheese-cloth in a basket-type centrifuge. The filtrate was applied in several portions to an Amberlite CG–120 column (H^+-form, 6.0ϕ × 60 cm), which was washed successively with distilled water and 4% ammonia. A plurality of bound vitamin B_6 was found to be distributed among effluents of showing pH 6 to 8.5. These effluents were combined and evaporated to a brownish syrup *in vacuo* below 40°C. The syrup was dissolved in appropriate volume of distilled water, and the materials remained insoluble were removed by centrifugation.

The resultant concentrate, which showed vitamin B_6 activity in the microbiological assay after either acid-hydrolysis or taka-diastase digestion, was submitted to a repeated ion-exchange chromatography for the purification of the compound in question as follows: first, on Dowex 1–X4 (OH-form, 3.5ϕ × 60 cm) with elution by distilled water and by 1 N acetic acid; then, on Dowex 50W–X8 (equilibrated with eluting buffer, 3.2ϕ × 45 cm) with elution by 0.1 M acetate buffer of pH 4.6; eventually, on Dowex 50W–X8 (H^+-form, 2.0ϕ × 30 cm) with elution by 4% ammonia; and finally, on Dowex 50W–X8 (equilibrated with eluting buffer, 0.9ϕ × 90 cm) with elution by 0.5 M ammonium formate buffer (pH 3.4) containing 40% ethanol. The active fractions, in respective chromatography, were combined and concentrated by evaporation *in vacuo*. An oily concentrate, obtained on evaporation of the active fractions from the last column, was applied to Toyo No. 51 filter paper as a streak and subjected to chromatography in acetone–water (9: 1). The paper was viewed under UV light and the faintly fluorescent area was cut out and eluted with ethanol–water (1: 1). The eluate after concentration *in vacuo*, was again subjected to paper chromatography in *n*-propanol–2% ammonia (2: 1). A single spot was detected at *Rf* 0.44 under UV light which corresponded to the compound of

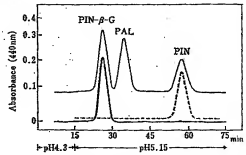

FIG. 1. Typical Chromatographic Pattern of Isolate and Its Hydrolysate on Aminex A–5 Column Taken with Semi-automated Apparatus.

The analytical conditions were the same as described previously[42]; bed resins, 0.9ϕ × 10 cm; flow rate, 50 ml/hr; buffer change, 15 min to pH 5.15; chart speed, 20 cm/hr. Hydrolysis of the isolate was performed at 120°C for 1 hr with 0.44 N HCl; ——, isolate; ——, hydrolysate; ——, a mixture of synthetic PIN-β-G, PAL and PIN.

giving a positive reaction with diazotized 5-chloroanilide reagent. Evaporation *in vacuo* of the alcoholic extract of the corresponding band left a faintly yellowish syrup (yield, 20 mg).

Chromatographic behavior and analyses

Figure 1 shows typical chromatographic patterns of the isolate in question and its hydrolysate taken with the semi-automated analyser system.[41] The isolate gave a single peak at elution position just preceding the PAL peak, but coincident with that of synthetic PIN-β-G; this singleness of the chromatogram rules out the possibility of the contamination with any detectable amount of such impurities as PIN, PAL, and PAM and their phosphate. On hydrolysis with 0.44 N HCl at 120°C for 1 hr, the isolated compound gave no peak but a mere residue at the position eluted before hydrolysis, instead a major peak at the position where free PIN emerges.

Table I summarizes the results of typical analyses by thin-layer chromatography of the isolate and its hydrolysate. The isolate gave *Rf* values identical to those observed with synthetic PIN-β-G. The hydrolysate contained, besides PIN, a sugar component giving a positive reaction with cysteine-sulfuric acid

TABLE I. *Rf* VALUES OF ISOLATE AND ITS HYDROLYSATE ON THIN-LAYER CHROMATOGRAM

An aqueous solution containing an appropriate amount of the isolate or its hydrolysate was applied to the Kieselguhr plate previously activated at 140°C, and then, chromatographed with the following developing solvents: I, chloroform–methanol (3: 2); II, *n*-buthanol–acetic acid–water (8: 2: 1); III, isopropanol–2% ammonia (2: 1); IV, chloroform–methanol (2: 1); V, *n*-buthanol–acetic acid–water (3: 2: 2); VI, *n*-propanol–2% ammonia (2: 1). Spots for PIN and sugar were visualized by spraying the plate with diazotized 5-chloroanilide and anthrone-sulfuric acid reagents, respectively.

Compound	Solvent system			Compound	Solvent system		
	I	II	III		IV	V	VI
Isolate	0.53	0.19	0.58	Hydrolysate	0.49	0.48	0.57
Hydrolysate	0.91	0.47	0.69	Glucose	0.48	0.49	0.57
Pyridoxine	0.91	0.47	0.70	Fructose	0.35	0.36	0.48
PIN-5′-phosphate	0.03	0.13	0.09	Mannose	0.48	0.45	0.54
PIN-5′-β-glucoside	0.52	0.20	0.58	Galactose	0.47	0.46	0.50

reagent.[13] In each chromatogram, a single anthrone-sulfuric acid-positive spot with *Rf* value and the color of glucose was detected (Table I). In the presence of boric acid, the isolate failed to give such a positive reaction with 2,6-dichloroquinone chlorimide as shown in its absence.[7] Since this test requires the presence of the free phenolic hydroxyl group in vitamin B_6 moiety, and added boric acid forms a strong complex across the phenolic hydroxyl group and the 4-hydroxymethyl group of the vitamin,[13] the negative test infers that the isolate should comprise glucose moiety bound to the 5-hydroxymethyl group of PIN.

Stoichiometry of PIN and glucose liberation by β-glucosidase action

The isolate was found highly vulnerable to the action of β-glucosidase (sweet almond) but least to that of α-glucosidase (baker's yeast). These results are in sharp contrast to the situations previously found with PIC-α-G produced in rat liver homogenates[9] and with PIN-α-G formed by intact cells of *Sarcina lutea*,[14] but consistent with the notion that β-glucosidic linkage to PIN is involved in the isolate. Identities of the products with PIN and D-glucose were established by the chromatographic method described in Table I. Further proof that the isolate is 5′-D-glucoside of PIN was provided from the stoichiometry of

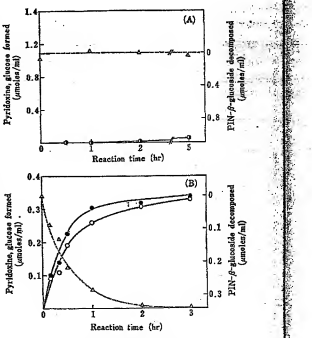

FIG. 2. Stoichiometric Relationship between Pyridoxine and Glucose Liberation from Isolate by α-Glucosidase (A) and β-Glucosidase Actions (B).

Details of the experimental conditions were given in METHODS: ●—●, PIN; ○—○, glucose; △—△, isolate.

the β-glucosidase reaction. Figure 2 shows the results of typical time-course experiments. The amounts of the isolate remained intact and of PIN released were determined simultane-

ously on the semi-automated analyser system.[4] The enzymatic reaction proceeded rapidly for the first 60 min and completed within 3 hr, at which an equimolar amount of PIN and D-glucose was produced with an equivalent consumption of the isolate. At the initial stages of the reaction, the PIN liberation appeared not to be exactly parallel with that of glucose, while keeping pace with the substrate consumption at every stage of the reaction. Some of this inconsistency may be ascribable to β-glucosidase-catalyzed transglucosidation between each other molecules of the substrate.

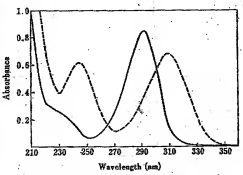

FIG. 3. Ultraviolet Absorption Spectra of Isolated PIN-β-G in 0.1 N NaOH (broken line) and 0.1 N HCl (solid line).

UV and NMR spectra

The UV absorption spectra of the isolate are given in Fig. 3. It showed absorption maxima at 246 and 310 nm in 0.1 N NaOH and at 292 nm in 0.1 N HCl with spectral features closely resembling with those of PIN. This supports the interpretation that either 4- or 5-hydroxymethyl group is involved in the linkage to D-glucose; binding of D-glucose to either 1- or 3-position of PIN should cause considerable alteration in the spectrum.

Figure 4 shows the NMR spectrum of the isolate in CD_3OD. A singlet at δ 7.78 is attributable to one proton of the 6-position of PIN. Sharp signals at δ 4.86 and 4.62 are assigned to 4- and 5-methylene protons respectively. A doublet at δ 4.22 (J=7 Hz) is ascribed to the β-anomeric proton of PIN-bound glucose by analogy with methyl-β-D-

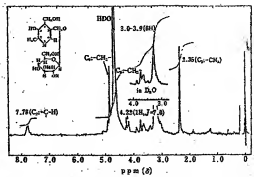

FIG. 4. NMR Spectrum of Isolated PIN-β-G in CD_3OD (3 mg/0.4 ml) at 90 MHz.

glucoside and with authentic PIN-β-G and from dissimilarity with PIC-α-G[9] and with PIN-α-G.[14] Signals at δ 3.5 to 3.9 are attributable to the hydroxymethyl protons in the glucose moiety; the signals for the other protons in the glucose moiety were hidden behind the strong CH_3OH signal and became detectable when the specimen was dissolved in D_2O. A sharp singlet at δ 2.35 is assigned to the methyl protons on the 2-position of PIN.

DISCUSSION

One of the bound forms of vitamin B_6 occurring in rice bran was isolated by repeating chromatographic purification. The analytical results so far obtained for the isolated compound were all compatible with the chemical structure of 5′-O-(β-D-glucopyranosyl)pyridoxine, represented herein by PIN-β-G. In addition, no essential difference could be detected in spectral features between the natural PIN-β-G and its synthetic counterpart.

Since Whitby first reported riboflavinyl glucoside,[15] a number of glycoside derivatives of B vitamins have been reported.[16~18] Among those of vitamin B_6 derivatives prepared by enzymatic procedures are PIN-α-G found in the filtrate of PIN-fortified culture medium of *Sarcina lutea*,[14] and PIC-α-G and -α-maltoside both formed in rat liver homogenates.[9,19] No clear evidence however has been presented for

the natural occurrence of any of these glycosides. PIN-β-G concerned in this paper is therefore the first proven to occur naturally, *e.g.* in rice bran, among this group of glycosides, although its physiological function or role in metabolism, being far beyond the scope of the present study, remains further investigated.

Fears were once entertained that PIN-β-G might be an artificial product formed by the action of endogenous enzymes in the course of the rice bran extraction which was effected as the first step in the isolation procedure. However, these fears were proven completely baseless. PIN-β-G was found to occur in not only the alcoholic extracts of raw (unstabilized) rice bran but also the aqueous extracts of autoclaved (stabilized) bran much more than in the aqueous extracts of raw bran.

The quantity of B vitamins in various foods and feeds has been well established by various assay procedures. The results reported in the literature are however often controversial and do not necessarily represent the availability or the amount of vitamins to be actually absorbed in animal. Among the B vitamins derivatives known by their diminished availability, if not nil, are niacin bound in a polysaccharide or to protein-niacytin,[20] folate in polyglutamate forms,[21] and biotin bound to avidin—the component responsible for the production of "egg white injury".[22]

The determination of the amount of B_6 vitamers has been achieved mostly by microbiological method using *Saccharomyces carlsbergensis* as test organism and following vigorous hydrolysis. Obtained data should undoubtedly represent a total amount of free and bound B_6 vitamers including PIN-β-G herein identified or other poorly available derivatives. PIN-β-G was first considered to be appended to this group of poor availability. This hypothesis was set off because the enzyme which splits off the glucose moiety, β-glucosidase, in the intestinal epithelium exhibits optimal activity at acidic side rather remote from the luminal pH,[23] and hence there was much possibility that this very nature is limiting the availability of PIN-β-G. However, the hypothesis is hardly tenable in view of the results obtained in rat feeding studies and *in vitro* transport experiments,[24] which demonstrate that the experimental animals are endowed with capability of utilizing PIN-β-G as vitamin B_6 or, more precisely, of converting it into PIN so as to alleviate pyridoxine deficiency lesions.

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Formation of 5'-*O*-(β-D-Glucopyranosyl) pyridoxine in Soybean Seedlings and Suspension Cells Cultured in the Presence of Pyridoxine

Yukio SUZUKI and Kei UCHIDA

A large accumulation of a pyridoxine derivative was observed in soybean seedlings cultured in the presence of pyridoxine in the dark. The derivative was purified from the extracts of seedlings by column chromatography on Dowex 50W-X8, preparative paper chromatography, gel filtration on Sephadex G-10, DEAE-cellulose column chromatography, and isolated as the crystalline hexaacetate. The derivative was identified as 5'-*O*-(β-D-glucopyranosyl) pyridoxine on the basis of elemental analyses, UV, ^{1}H-NMR and ^{13}C-NMR spectra, electrophoretic mobilities, color reaction with 2,6-dichloroquinone 4-chloroimide in the presence of boric acid, and the hydrolysis products by β-glucosidase from almond. The derivative accumulated in a high yield in soybean cultured cells grown in a medium containing pyridoxine. The pyridoxine activity of the derivative for the growth of *Saccharomyces carlsbergensis* was less than 10% (molar ratio) of that of pyridoxine.

Key words: soybean seedlings, soybean suspension cells, 5'-*O*-(β-D-glucopyranosyl) pyridoxine, β-glucosylpyridoxines, microbiological activity.

INTRODUCTION

Vitamin B_6 occurs naturally in plants largely in conjugated forms of pyridoxine and glucose.[1,2] Its major form in rice bran[2] is 5'-*O*-(β-D-glucopyranosyl) pyridoxine. Tsuji *et al.*[3] reported that chemically synthesized 5'-*O*-(β-D-glucopyranosyl) pyridoxine was biologically available as good as pyridoxine to vitamin B_6-deficient rats and was permeable in its intact form to everted sacs of rat small intestine. Trumbo *et al.*[4] reported the

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controversial results that bioavailability of 5'-*O*-(β-D-glucopyranosyl) pyridoxine against B_6-deficient rats was 10 to 34% of pyridoxine. To examine this discrepancy, a large amount of 5'-*O*-(β-D-glucopyranosyl) pyridoxine is required. Previously,[5,6] we reported that two β-glucosylpyridoxines [5'-*O*-(β-D-glucopyranosyl) pyridoxine and 4'-*O*-(β-D-glucopyranosyl) pyridoxine] were formed after incubation of cellobiose and pyridoxine with wheat bran β-glucosidase (cellobiase). During our investigations on the formation of these glucosylpyridoxines in several cereal seedlings cultured on a pyridoxine solution, a large amount of accumulation of a β-glucosylpyridoxine-like compound was observed in soybean seedlings. Herein, we report the isolation and characterization of a β-glucosylpyridoxine from soybean seedlings, its formation by soybean suspension cultures, and its microbiological activity.

EXPERIMENTAL

Materials.

Barley seeds (*Hordeum vulgare* L. var. nudum cv. Kikaihadaka), wheat seeds (*Triticum aestivum* L. cv. Shirasagi), rice seeds (*Oryza sativa* L. cv. Taichung 65, nonwaxy and *Oryza sativa* L. cv. Norin 18, nonwaxy) and soybean seeds (*Glycine max* L. Merr. cv. Tamanishiki) were harvested in the experimental field of our Institute. Yeast α-glucosidase and almond β-glucosidase were purchased from Boehringer Mannheim, GmbH, Mannheim, Germany. The crystalline α-glucosidase from the mycelia of *Mucor javanicus* was prepared, as described previously.[7] Pyridoxine·HCl, pyridoxal·HCl, and pyridoxamine·2HCl were obtained from Nacalai tesque, Inc., Kyoto, Japan.

Methods.

Assays of pyridoxine and its derivative in seedlings. Seeds were soaked in a disinfectant solution (0.25% Homai WP, Nippon Soda Co., Ltd., Tokyo, Japan) for 15 min at 40°C and left for 5 hr at room temperature. After the seeds were thoroughly rinsed in running water, they were sown in vermiculite placed on plastic plates. The seeds were germinated and cultured at 20-27°C in the dark on water or a 10 mM pyridoxine·HCl solution (neutralized to pH 4.8 by NaOH). Seedlings were harvested after cultivation, washed, heated for 15 min in 0.02 M acetate buffer, pH 4.8, in a boiling water bath, and then homogenized. The homogenate was again heated for 15 min in a boiling water bath. One volume of ethanol was added to the homogenate, and centrifuged. The supernatant solution was concentrated at

30°C *in vacuo*. The concentrate was applied to paper chromatography (PPC), using n-butanol-pyridine-water (6 : 4 : 3, v/v/v) (solvent A). Each fluorescent spot of pyridoxine and its derivatives was cut out, and eluted with 0.1 M phosphate buffer, pH 7.0, for 3 hr at 37°C. The fluorescent intensity of the buffered eluate was measured with a spectrofluorometer attached to a Hitachi model EPS-3T automatic recording spectrophotometer (excitation at 325 nm; emission at 400 nm). Pyridoxine and its derivatives were also measured colorimetrically by the diazotized *p*-aminoacetophenone method with a slight modification.[8] The values obtained by the two methods were similar. The content of pyridoxine derivative was expressed as pyridoxine equivalent.

Assays of pyridoxine and its derivatives in cultured cells. The sterilized soybean seeds were put on the basal agar medium of Murashige and Skoog containing 3% sucrose and 4.52 μM 2,4-dichlorophenoxy acetic acid. After about 3 weeks at 25°C in the dark, the calluses induced from the germinating seeds were transferred on the agar medium, and successively subcultured more than 20 times at intervals of 3 weeks, and then used for experiments. A piece of the soybean callus was grown on 50 ml of the above agar medium with or without 10 mM pyridoxine·HCl (neutralized to pH 5.7 by NaOH) in 100 ml flask at 25°C in the dark. Also, the callus was grown on 50 ml of the Murashige and Skoog's liquid medium containing sucrose, 2,4-dichlorophenoxy acetic acid and 10 mM pyridoxine in 500 ml shaking flasks at 25°C in the dark under shaking conditions. At intervals of 3-4 days, calluses (or cells) in 3 flasks were harvested, and the contents of pyridoxine and its derivative in calluses (or cells) was analyzed, as described above.

Paper chromatography and thin layer chromatography. The vitamin B_6 compounds on chromatograms were detected under an ultraviolet ray lamp (2537 Å, filter). Sugar spots on paper chromatogram were detected by the silver nitrate dip method,[9] while the spots on this layer chromatogram were visualized by spraying the sheet (kieselgel 60 F_{254}) with 5% H_2SO_4 in ethanol, followed by heating at 100°C.

Paper electrophoresis. Electrophoresis was carried out at 400 V for 1 hr at room temperature on a Toyo No.51 filter paper (width 12 cm x length 26 cm) with 0.01 M sodium phosphate buffer, pH 7.0, using a Toyo paper electrophoretic apparatus (Tokyo, Japan). Electrode buffer, 5% KCl; agar bridge, 2% agar containing 5% KCl.

Hydrolysis by glucosidases. The enzymatic hydrolysis of the isolated pyridoxine derivative was carried out in a reaction mixture (1 ml) containing pyridoxine compound (4 mg), 4-5 units of α- or β-glucosidase and 50 μmoles of buffer in a final volume of 1 ml. Yeast α-glucosidase was incubated in phosphate buffer, pH 7.0. Almond β-glucosidase and *M. javanicus* α-glucosidase were incubated in acetate buffer, pH 5.0. The incubation was performed at 37°C for 20 hr. The reaction was stopped by immersion of the reaction mixture in a boiling bath for 5 min. An aliquot of each reaction mixture was applied to PPC, TLC, and high performance liquid chromatography (HPLC) to estimate and identify hydrolysis products. The amounts of sugar and pyridoxine liberated enzymatically were determined by the method of Nelson[10] and the diazotized *p*-aminoacetophenone method,[8] respectively.

Instrumental analyses. UV absorption spectra were measured with a Hitachi Perkin-Elmer model 139 spectrophotometer. Infra-red (IR) absorption spectra were measured in a KBr tablet with a Hitachi IR spectrophotometer model 260-30. ^{1}H- and ^{13}C-NMR spectra were taken on a JEOL JNM-FX-100 spectrometer at 25°C in D_2O at 100 MHz. The spectra of acetyl compound were obtained with $CDCl_3$ as solvent. Tetramethylsilane was used as an internal standard.

Microbiological assay of pyridoxine derivative. The pyridoxine activity of the isolated pyridoxine derivative on microorganisms was assayed by the method of Fukui *et al.*,[11] using *Saccharomyces uvarum* IFO 0565 (*Saccharomyces carlsbergensis* strain 4228, ATCC 9080) as a test microorganism.

RESULTS

1. *Formation of β-glucosylpyridoxines in several cereal seedlings cultured on a pyridoxine solution.*

The metabolism of exogeneous pyridoxine was investigated in several cereal seedlings cultured in the dark (Table 1). Two derivatives (I and II) of pyridoxine were tentatively identified as 5'-O-(β-glucosyl) pyridoxine and 4'-O-(β-glucosyl) pyridoxine, respectively, by comparing several properties with the authentic β-glucosylpyridoxines synthesized from cellobiose and pyridoxine by wheat bran β-glycosidase; *Rf* values, liberation of glucose and pyridoxine by almond β-glucosidase, and UV spectra in both borate solution and phosphate buffer were identical with authentic samples. The two glucosylpyridoxines were formed in a 1 : 1 molar ratio in seedlings of

Table 1. Formation of pyridoxine derivatives I and II in several cereal seedlings cultured in the presence of pyridoxine.

Days after imbibition	PN	I	II
	(μg/grain)		
Triticum aestivum L. cv. Shirasagi			
4	37	3	3
6	102	11	13
9	150	24	30
12	208	50	50
Hordeum vulgare L. var. nudum cv. Kikaihadaka			
4	40	3	3
7	85	10	11
14	240	36	40
Hordeum vulgare L. var. nudum cv. Kikaihadaka*			
7	110	12	12
14	380	70	78
Oryza sativa L. cv. Taichung 65			
8	16	7	±
11	57	19	+
14	119	41	6
Oryza sativa L. cv. Norin 18			
14	138	43	7
Glycine max L. Merr. cv. Tamanishiki			
7	210	400	ND
10	627	1200	ND
13	550	1516	ND

PN: pyridoxine.
* : Seedlings were cultured in a temperature-controlled chamber (20°C) in the light (7800 luxes).

wheat and barley. In rice seedlings, 5'-*O*-(β-glucosyl)-pyridoxine was the major metabolite, and 4'-*O*-(β-glucosyl)-pyridoxine as the minor one was also detected at the later stages of germination. A large amount of accumulation of 5'-*O*-(β-glucosyl) pyridoxine was observed in soybean seedlings. The amount of the derivative I accumulated was much larger than that of pyridoxine at all stages of germination. Formation of α-glucosylpyridoxines was not observed in any cereal seedlings tested.

2. *Isolation of pyridoxine derivative I in soybean seedlings cultured in the presence of pyridoxine.*

The identity of 5'-*O*-(β-glucosyl) pyridoxine was further confirmed by isolating the pure pyridoxine derivative I from soybean seedlings cultured in the presence of pyridoxine. The concentrate (600 ml) of the extract from 10-day-old seedlings (4 kg) was prepared as described in EXPERIMENTAL. The concentrate was adjusted to pH 2.0 after dilution with 0.01 N HCl, divided into four portions, and each was applied to a separate column (5.6×

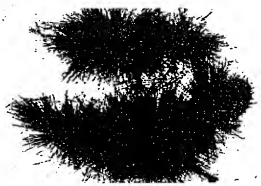

Fig. 1. Photomicrograph of crystalline hexaacetate (API) of pyridoxine derivative PI.

90 cm) of Dowex 50W-X8 (H^+) at 4°C. After the columns were washed successively with 0.01 N HCl and water, pyridoxine and derivative I were eluted with 1% ammonia water at 4°C. The fractions containing derivative I were combined and concentrated under reduced pressure. The concentrate was applied to the preparative paperchromatograph (PPC) with solvent A. The band of derivative I was cut out, eluted with acetic acid solution, pH 4.8, concentrated and re-applied to PPC. The second PPC was developed two times by ascent in iso-propanol-ethanol-water (7 : 1 : 2, v/v/v). After appropriate sectioning and elution, derivative I solution was concentrated and applied to the first gel-filtration on a Sephadex G-10 column (5.6×90 cm) at 4°C, followed by elution with water. The desired eluent was lyophilized to give derivative I as a powder. The powder dissolved in water was applied to the first DEAE-cellulose (OH^-) column chromatography (5.6×42 cm) at 4°C. After elution with water, the eluate of derivative I was lyophilized, and rechromatographed successively on the second gel-filtration on a Sephadex G-10 column, the second DEAE-cellulose column, and the third gel-filtration on Sephadex G-10 column, followed by elution and lyophilization. The colorless powder of purified derivative I was dried *in vacuo* (yield of the purified derivative PI was 9.5 g). The powdered derivative PI (600 mg) was acetylated with pyridine and acetic anhydride. The acetylated derivative (API) was recrystallized five times from 20% ethanol solution to give white needle crystals (yield 670 mg) (Fig. 1). Its melting point was 85-87°C. The results of elementary analyses of the derivatives PI and API were as follows. PI : Found ; C, 49.67 ; H, 6.39 ; N, 4.27%. Calcd. for $C_{14}H_{21}NO_8$: C, 50.76 ; H, 6.34 ; N, 4.23%. API : Found ; C, 53.39 ; H, 5.69 ; N, 2.29%. Calcd. for $C_{26}H_{33}NO_{14}$: C, 53.51 ; H, 5.70 ; N, 2.40%. The lyophilized powders of the derivative PI and its crystalline acetyl compound API were used for the characteriza-

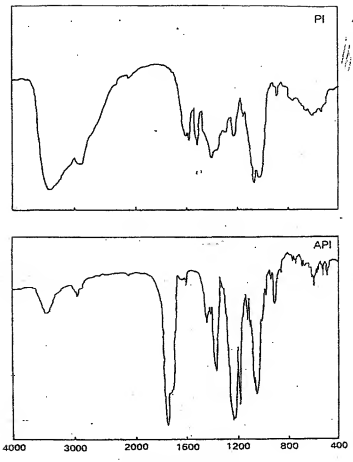

Fig. 2. Infrared spectra of pyridoxine derivative PI and its hexaacetate.

tion.

3. *Characterization of pyridoxine derivative (PI) and its acetyl compound (API).*

Derivative PI was hydrolyzed completely to glucose and pyridoxine in a molar ratio of 1 : 1 by almond β-glucosidase, but not at all by α-glucosidases from both yeast and *M. javanicus*. Glucose was identified as a single reducing product of hydrolysis on TLC with n-propanol-2% ammonia water (2 : 1, v/v) as solvent, and also identified by the formation of gluconic acid on paper chromatogram developed with solvent A after treatment with

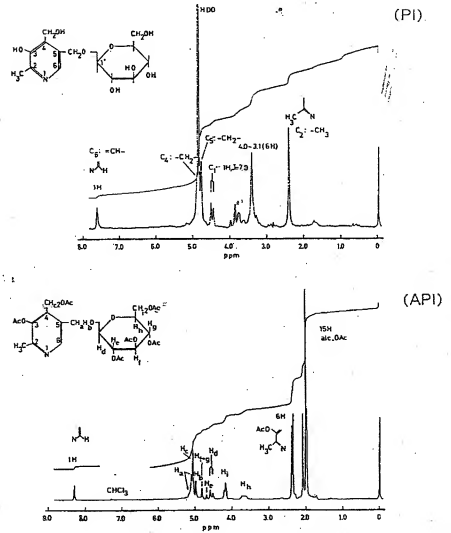

Fig. 3. ^{1}H-NMR spectra of pyridoxine derivative PI and its hexaacetate.

glucose oxidase. Moreover, glucose gave a single peak at an elution position identical with that of an authentic specimen by HPLC with a Waters carbohydrate analysis column (0.4×30 cm) developed with CH_3CN-water (9 : 1, v/v). Pyridoxine released was identical with an authentic specimen in UV spectra and *R*f values on paper chromatograms developed with several solvent system of n-amylalcohol-acetone-water (2 : 1 : 2, v/v/v), iso-amylalcohol-pyridine-water (2 : 1 : 2, v/v/v) and n-butanol saturated with 0.2

Table 2. Chemical shift in ^{13}C-NMR of pyridoxine derivative (PI) and its hexaacetate (API).

Carbon number*	PI (ppm)	API (ppm)	
Pyridoxine carbon			
C-2 (s)	148.4	144.9	
C-2' (q)	18.8	19.5	
C-3 (s)	162.4	153.2	
C-4 (s)	140.8	135.8	
C-4' (t)	59.0	56.9	
C-5 (s)	133.9	129.8	
C-5' (t)	68.9	68.9	
C-6 (d)	130.5	147.3	
Transferred glucose carbon			
C-1' (d)	104.0	99.2	
C-2' (d)	75.6	71.1	
C-3' (d)	78.6	72.8	
C-4' (d)	72.3	68.2	
C-5' (d)	78.3	71.9	
C-6' (t)	63.4	63.4	
CH_3 ×6			
—C=O		20.5	
C=O		170.6 170.2	170.1
		169.3 169.2	168.5

* : Carbon number assignments of derivative PI (Fig. 3).
s, singlet; d, doublet; t, triplet; q, quartet.

M acetate buffer, pH 5.0, but different from pyridoxal and pyridoxamine in *Rf* values. Also, pyridoxine was confirmed by HPLC on LiChrosorb NH_2 column (10 μm, 0.4×25 cm) with CH_3CN-water (3 : 1, v/v). In paper electrophoresis, derivative PI migrated on the paper chromatogram at a similar rate to pyridoxine toward the cathode. The UV spectra of derivative PI in 0.1 N HCl, 0.1 M phosphate buffer, pH 7.0, and 0.1 N NaOH closely resembled with those of pyridoxine. Derivative PI gave a blue color with 2, 6-dichloroquinone chloroimide, but not in the presence of boric acid. Derivative PI also, showed the same UV spectrum (λ_{max} 294 nm) without any shoulder at 326 nm as that of pyridoxine, when saturated with boric acid at pH 6.8. The IR spectra of derivatives PI and API are shown in Figure 2. These data suggest that derivative PI is β-glucosylpyridoxine in which N-1 and both the hydroxy groups on C-3 and C-4' in pyridoxine moiety are unsubstituted. The fine structures of derivatives PI and API were confirmed with ^{1}H- and ^{13}C-NMR spectra (Fig. 3 and Table 2). Signal assignments were based upon the data reported for pyridoxine[12] and methyl glucopyranoside.[13,14] From these findings, derivative PI and API were identified as being 5'-O-(β-D-glucopyranosyl) pyridoxine and its hexaacetate, respectively.

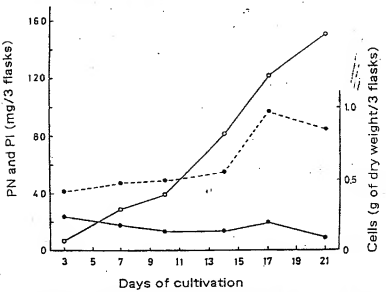

Fig. 4. Changes in content of pyridoxine derivative PI in soybean cells grown on sucrose liquid medium with 10 mM pyridoxine in shaking culture.
●——● : PN (pyridoxine)
○——○ : PI
●·····● : cells

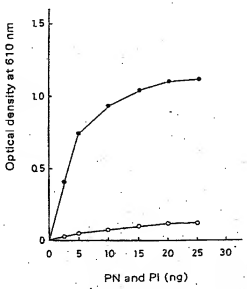

Fig. 5. Pyridoxine activity of derivative PI for growth of Saccharomyces carlsbergensis.
●——● : PN (pyridoxine)
○——○ : PI

4. *Formation of pyridoxine derivative PI by soybean suspension cultures.*

Suspension cultured cells of soybean had a high activity to form derivative I (Fig. 4). After 21 days of cultivation, the content of derivative I in cells was about 50% of pyridoxine added to the liquid medium. On prolonged cultivation (30 days), 80% of pyridoxine added was converted into derivative I in cells. Very little of derivative I was present in the culture fluid at all stages of cultivation.

5. *Microbiological activity of derivative PI.*

As shown in Fig. 5, the pyridoxine activity of derivative PI for the growth of *Saccharomyces carlsbergensis* was not more than 10% of that of equivalent mole of pyridoxine after a 16-hr incubation.

DISCUSSION

5'-*O*-(β-D-Glucopyranosyl) pyridoxine was isolated in a crystalline form of its hexaacetate from soybean seedlings cultured on a pyridoxine solution. To our knowledge, this is the first case in which 5'-β-glucosylpyridoxine was obtained as crystals, although crystals of hexaacetate·HCl of 5'-α-glucosylpyridoxine[15] and hexaacetate of 4'-β-glucosylpyridoxine[6] were obtained from the culture broth of *Sarcina lutea* and incubation mixture with wheat bran β-glucosidase, respectively. The formation of 5'-*O*-(α-glucopyranosyl) pyridoxine and 4'-*O*-(α-glucopyranosyl)-pyridoxine was first found by Ogata *et al.*[8,16] in the culture broth of *Sarcina lutea* which was grown in the sucrose medium with pyridoxine. Subsequently, several glycosidases such as α-glucosidases from *Aspergillus niger*,[17] *M. javanicus*,[17,18] *Micrococcus* sp.,[19] and β-glucosidase from wheat bran,[5,6] β-galactosidase from *Escherichia coli*,[17,20] α-mannosidase from *Canavalia ensiformis*,[21] and β-N-acetylglucosaminidases from *A. oryzae*[21] were observed to transfer the glycosyl residue from disaccharides (or nitrophenyl glycosides) to hydroxymethyl groups at C-4' and C-5'of pyridoxine to yield two glycosylpyridoxines. A particulate enzyme in seedlings of *Pisum sativum* L. cv. Kinusaya was reported to be capable of transferring the glucose moiety of uridine diphosphoglucose only to the 5'-, but not 4'-hydroxymethyl group of pyridoxine.[22] These data on the glycosylpyridoxine-forming enzymes suggest that the two β-glucosylpyridoxines in seedlings of wheat and barley may be formed by β-glucosidase, and 5'-*O*-(β-glucopyranosyl)-pyridoxine in soybean seedlings and cultured cells by uridine diphosphoglucose-pyridoxine glucosyltransferase.

α-Glucosylpyridoxine has been reported to show approximately 20% of microbiological activity of equivalent mole of pyridoxine for *Saccharomyces carlsbergensis* after 24 hr of incubation.[23] The activity of 5'-O-(β-D-glucopyranosyl) pyridoxine was less than 10% after 16 hr of incubation.

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